

Investigation of Large Intramolecular Movements within Metmyoglobin by Rayleigh Scattering of Mössbauer Radiation (RSMR)

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This paper reports Rayleigh scattering experiments on metmyoglobin crystals and freeze dried myoglobin which was exposed to air with different partial pressure of water vapor. While dry myoglobin shows no fluctuations between conformational substates such “breathing modes” are rarely seen in water covered myoglobin. Larger amounts of water increase the average mean square displacements. In crystals the dynamic behaviour is hindered by the crystal packing. The results are analysed by a theory describing the motion within the molecule by a Langevin equation with restoring forces corresponding to a square well potential.

1. Introduction

Recent theoretical and experimental studies on proteins proved that these molecules are rather flexible systems. Smaller or larger portions of the molecule can experience large mean square displacements. It is now generally accepted that such a molecule has a large number of conformational substates or microstates. This means that the structure of all molecules is perceived as identical if one uses only limited resolution (*i.e.* 0.2 Å [1]) but becomes slightly different at higher resolutions. Fluctuations between conformational substates can be thermally activated. However, up to now there exists no detailed knowledge on the physical nature of the conformational substates or microstates. A review of the investigations of the structural dynamics of proteins is given by Gurd *et al.* [2].

Besides other techniques, X-ray diffraction and Mössbauer spectroscopy reveals some details of the motions of the atoms of the molecules [1], [3–6]. Both techniques yield the mean square displacement, $\langle x^2 \rangle$, of atoms within the molecule via the Debye Waller factor in case of X-ray diffraction and

through the Lamb Mössbauer factor in case of nuclear gamma resonance absorption. There are, however, two remarkable differences between the information obtained from these techniques: First of all X-ray diffraction yields informations on all atoms within the molecule while nuclear gamma resonance absorption gives only informations on the Mössbauer atom which is normally the isotope ^{57}Fe in the case of proteins. Secondly the time dependence of the two techniques is different. X-ray diffraction is a prompt process where no time dependence is involved. Therefore, the derived $\langle x^2 \rangle$ of the atoms include both dynamical displacements and possible static disorder such as lattice disorder in crystals [7, 1]. By contrast the nuclear resonance absorption has a builtin “clock” reflecting the lifetime, τ_N , of the involved excited nuclear level. Therefore, the displacement $\langle x^2 \rangle$ measured by nuclear gamma resonance contains only motional contributions which are faster or comparable to τ_N , while static disorder contributions are absent. An advantage of the nuclear gamma resonance technique is the fact that the $\langle x^2 \rangle$ of the ^{57}Fe can easily be studied as a function of temperature which helps to understand the mechanism of motion. Specifically it yields information on the activation energy of the fluctuations between conformational substates.

The mean square displacement of atoms in solids or molecules can also be studied by Rayleigh

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scattering of Mössbauer radiation (RSMR). This technique is in some sense a combination of X-ray diffraction and nuclear gamma resonance absorption. RSMR technique employs very often the 14.4 keV radiation of an excited ^{57}Fe nucleus. The extremely monochromatic Mössbauer line emitted in this transition is Rayleigh scattered by the electrons of the substance under investigation. Because of the extreme monochromaticity of the Mössbauer line it is rather easy to separate the elastic and inelastic contribution in the scattered intensity, by contrast to the X-ray method where such a separation is not readily feasible. The RSMR technique employs an ancillary resonance absorber which is successively introduced either before or after the sample under study. This way one can determine the reduction of the intensity which the Mössbauer line suffers by inelastic processes in the sample. It is one great advantage of this method that it is not confined to molecules which contain a Mössbauer isotope [8–10]. If the sample under investigation is a powder, a frozen solution or a mixture of statistically oriented small crystals the RSMR behaves as an incoherent process. Coherent contributions which may come from nearest neighbour correlations as in fluids can be averaged by using sufficiently large opening angle of the detector. The scattering on different atoms in the sample can then be treated as uncorrelated. As a consequence RSMR is not sensitive to static disorder. RSMR experiments on samples as described above yield informations on $\langle x^2 \rangle$ average over all atoms of the sample. Due to the nature of Rayleigh scattering the $\langle x^2 \rangle$ is weighted by the angular factor $(\sin \vartheta/\lambda)^2$ as in the case of the Debye Waller factor of X-ray diffraction where 2ϑ is the scattering angle and $\lambda = 0.86 \text{ \AA}$ in the case of ^{57}Fe . The use of a Mössbauer absorber in the analysis of elastic fraction of the radiation introduces the time τ_N as limit for the characteristic time of the motions to which RSMR is sensitive.

Preliminary experiments to study the fluctuation between conformational substates in metmyoglobin by RSMR are described in [11, 12]. Since one can use myoglobin solutions as well as crystalline or freeze dried samples RSMR is especially suitable to study the influence of water on the flexibility of the molecules. In the present contribution metmyoglobin samples with varying water contents are investigated. RSMR techniques were applied in the temperature range from 77 K to room temperature.

2. Materials and Methods

Four samples of metmyoglobin from sperm whale were investigated. The sample preparation started from freeze dried material. The dry myoglobin for a) was stored about 3 days in air 37% saturated with water vapour. Then it was sealed in a vacuumtied sample holder. Sample b) was prepared in the same way but was kept for 3 days in contact with 94% water vapour saturated air. Sample c) contained a water solution of myoglobin with a concentration of 29.6 weight percent of myoglobin. As sample d) a large number of small myoglobin crystals with random orientations was used. The surface of the crystals was carefully cleaned from excessive liquid so that the water content of the sample was determined exclusively by the crystal water. The crystals were grown at pH 6.1 from 3.75 M ammonium sulfate solution.

A radiation source of 100 mCi of ^{57}Co embedded in Cr was used. The experimental linewidth of this source in combination with a thin iron absorber was about 0.35 mm/s. The analysis of the elastic fraction of the radiation before and after the sample was performed with a $\text{Li}_3\text{FeF}_6(\text{NH}_4)\text{F}_3(\text{FeF}_3)_2$ absorber. The iron content was 7 mg/cm². In order to destroy the resonance condition between source and absorber the source could be moved sinusoidally with a maximum velocity of about 100 mm/s. The scattered radiation was detected by a annular NaJ(Ta) scintillation counter. This makes it possible to detect at the same time all radiation scattered into a cone with an opening angle of 2ϑ , in order to increase the counting rate. In the present experiment the average scattering angle $\langle 2\vartheta \rangle$ was $12^\circ \pm 1.5^\circ$. An acceptance angle of 5° was used in order to obtain reasonable counting rate and to average over contributions of short range correlations, as mentioned in the introduction. The absolute value of the temperature of the sample was controlled to better than $\pm 2.5^\circ$. Details of the equipment are described elsewhere [13].

Experimental Results, Data Evaluation

For each experimental point the intensities $I_0(0)$; $I_\infty(0)$; $I_0(2\vartheta)$ and $I_\infty(2\vartheta)$ were determined. The indices 0 and ∞ specify the source velocity which was either 0 or 100 mm/s, corresponding to in-resonance or off-resonance conditions. The arguments in brackets refer to the position of the Möss-

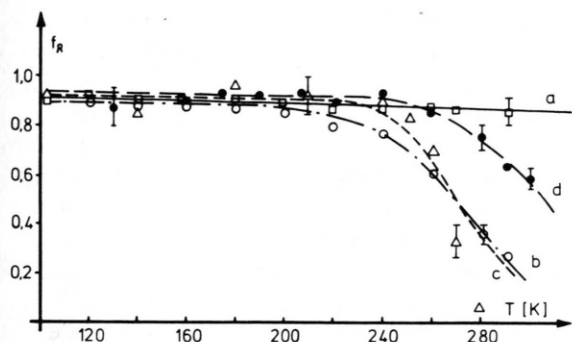


Fig. 1. Experimental f_R -values of the myoglobin samples at different temperatures. The lines give calculated values (compare text). Sample a: $\square$ and —; sample b: $\circ$ and ---; sample c: $\triangle$ and - - -; sample d: $\bullet$ and —; representative error bars are given at some points.

bauer absorber used as analyser. The notation (0) specifies a position between source and sample while (2 θ) indicates that the analysing absorber was placed between sample and detector. One then obtains easily

$$\frac{I_\infty(2\theta) - I_0(2\theta)}{I_\infty(0) - I_0(0)} = \gamma_R \exp(-Q^2 \langle x^2 \rangle_R) = f_R, \quad (1)$$

where $\langle x^2 \rangle_R$ is usually understood as the mean square displacement of the scattering atoms averaged over all atoms of the sample and $Q = \frac{4\pi}{\lambda} \sin \theta$ with $\lambda = 0.86 \text{ \AA}$ in the case of ^{57}Fe radiation. γ_R corrects for the contribution of Compton scattering and was calculated to be equal 0.96 (for details compare [12]). In the case of sample c) (myoglobin solution) and sample d) (myoglobin crystals) the samples contain large amounts of ammoniumsulfat and/or water. Therefore the contribution to the scattered intensity by the non myoglobin part of the sample has to be taken into account. Separate experiments on samples of pure water and 3.75 M ammoniumsulfat solution were performed. These experiments were used for a correction as described in [12]. Therefore, all f_R values given in the following figures refer to contributions of the myoglobin only. The f_R values of the four samples are given in Fig. 1.

Discussion

As already mentioned the structural dynamics of metmyoglobin has been previously investigated by

Mössbauer absorption spectroscopy [4–6]. The obtained $\langle x^2 \rangle_M$ -values can be understood in the following way [4]. One may distinguish two temperature regions. Below about 210 K the quantity $\langle x^2 \rangle_M$ of the iron atom has the same typical magnitude as is observed in other inorganic or organic iron compounds. It is essentially determined by the ordinary lattice vibrations in solids. Above 210 K a new motional contribution appears. The weight of this contribution increases with increasing temperature. This new motional degree of freedom can be attributed to fluctuations between conformational substates or as it is sometimes called in the literature to “breathing modes”. The iron atom labels this motion and allows its analysis. It should be mentioned that contributions of diffusion processes to $\langle x^2 \rangle_M$ can be neglected due to the crystalline nature of the sample. A comparison of Mössbauer [4] and X-ray diffraction data [1] has also shown that intermolecular motions play a minor role.

Fig. 2 gives the $\langle x^2 \rangle_R$ -values obtained for sample a) to d) by RSMR (curve a to d). These values were obtained from the experimentally determined f_R -values according to Eqn. (1). A comparison of these $\langle x^2 \rangle_R$ -values and the $\langle x^2 \rangle_M$ -values of [4] shows essentially the same gross temperature dependence. In the high temperature region $\langle x^2 \rangle_R$ grows rapidly due to the increase in the contribution from fluctuations between conformational substates. This growth is, however, much more pronounced than in the case of $\langle x^2 \rangle_M$. This can easily be understood if

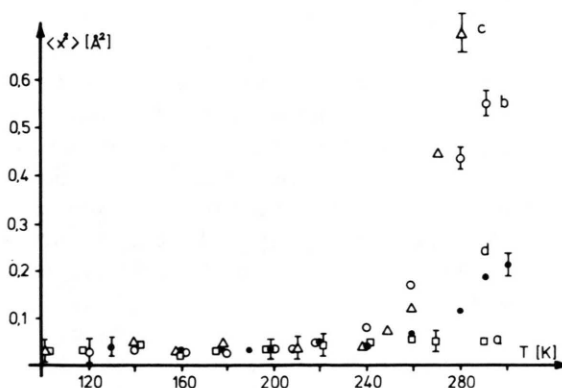


Fig. 2. Mean square displacement $\langle x^2 \rangle_R$ averaged over all atoms of myoglobin but corrected for the water content, calculated from f_R -values of Fig. 1. Sample a: $\square$; sample b: $\circ$; sample c: $\triangle$; sample d: $\bullet$.

we compare our results with the X-ray diffraction data of Frauenfelder *et al.* [1]. It is shown there that the myoglobin molecule contains regions with rather different flexibilities. The iron is located at a molecular position exhibiting a much lower flexibility than the average flexibility of the entire molecule. $\langle x^2 \rangle_R$ samples the average motions of all atoms while $\langle x^2 \rangle_M$ exhibits only the motion of the iron.

We now compare the $\langle x^2 \rangle_R$ -values of the crystalline sample (sample d)) with the $\langle x^2 \rangle_R$ -values of the sample a) prepared in air with rather low water content. Obviously no noticeable change of the behaviour of $\langle x^2 \rangle_R$ occurs for this sample in the high temperature region. In dry myoglobin no new motional degree of freedom appears at higher temperatures. Apparently, no fluctuations between conformational substates occur which have a characteristic time comparable or shorter than 10^{-7} s.

Curve b) of Fig. 2 gives the $\langle x^2 \rangle_R$ -values obtained with sample b) prepared in air nearly saturated with water vapor. The increase of $\langle x^2 \rangle_R$ above 210 K is much more pronounced than in the crystalline sample. Freeze dried myoglobin in a water vapor saturated atmosphere differs substantially from a solution of myoglobin in water. Diffusion processes of whole molecules should therefore be of no importance. It is more likely that the myoglobin molecules in crystals are hindered in their internal motions. The space limitation in the crystallographic unit cell to some extent imposes restrictions on the outer shape of the molecule. Fluctuations with drastic changes of the shape become impossible. In sample b) the molecules are less close packed as in crystals. Changes in the shape, therefore become easier. A comparison with the dry sample shows again that a water shell around the molecule needs to be present to make fluctuations possible. Curve c) of Fig. 2 refers to myoglobin solution. The extreme increase of $\langle x^2 \rangle_R$ with temperature starting very rapidly at about 230 K clearly demonstrates that in this case diffusion processes play an important role.

The discussion of the temperature dependence of $\langle x^2 \rangle_R$, $\langle x^2 \rangle_M$ or of the equivalent f_R , f_M helps to get some insight into the physical nature of the fluctuation processes in proteins. In [4] the simplest picture of conformational substates was given. There, the number of substates was confined to two.

In the gamma resonance absorption spectrum this "two state model" yields two Lorentzians, one Lorentzian with natural line width and one with broad line width corresponding to elastic and quasi-elastic absorption respectively. Within a protein molecule there should exist an extremely large number of conformational substates [1]. A natural extension of the two state model mentioned above should therefore give an absorption cross section for nuclear gamma resonance as a sum of a large number of Lorentzians with different broadened linewidths and of a Lorentzian with natural line width. The sharp Lorentzian line with natural width is always present, if the motion of the iron nucleus is confined to a region with a characteristic dimension which is comparable or similar than the wavelength λ used in the experiment.

One may also start from another picture for the motions of the atoms in the molecule due to fluctuations between conformational substates. Fragments i of the molecule with the masses m_i move relative to each other. This motion is driven by stochastic forces $F(t)$ of the thermal bath. The motion of one fragment with the mass m is damped by the interactions with other fragments. This is equivalent to say that each fragment moves in a liquid with the viscosity η . In crystals the motion has to be restricted to a certain volume. Therefore restoring forces have to act on each fragment in order to forbid unlimited diffusion. The picture represents the case of Brownian motions within a volume of limited extension. It is equivalent to the description of McCammon *et al.* [14] and Frauenfelder *et al.* [1] which call such a behaviour "fluid like" or "semi-liquid", respectively. A mathematical formulation of such a picture was recently given by Berg [15] for the case of DNA and by Shaitan and Rubin [16] for the explanation of Mössbauer data obtained on chromatophores.

The most simple calculation starts with a Langevin equation with a harmonic force [16]:

$$m \ddot{x}(t) + \gamma \dot{x}(t) + m \omega_r^2 x(t) = F(t), \quad (2)$$

where γ is a damping constant determined by the viscosity η and $m \omega_r^2 x(t)$ is the harmonic restoring force. Because of the stochastic nature of the driving force $F(t)$ one has to use correlation functions in order to calculate the mean square deviations. $\langle \Delta x^2(t) \rangle$, for a fragment which has started from some origin $x = 0$ at $t = 0$. In order to calculate the

value f_M in the case of Mössbauer effect Shaitan and Rubin [16] used the formulation of Singwi and Sjölander [17] for the cross section, σ_a , of nuclear gamma resonance absorption together with the Gaussian approximation for the Van Hove self correlation function.

We now want to apply this model for a quantitative understanding of our RSMR data. It is necessary to choose a proper restoring force which is representative for the behaviour of the majority of the atoms. From X-ray diffraction data [1] it was deduced that only a few atoms within the myoglobin molecule move harmonically. There exist atoms which apparently move in a rather steep potential. The motion of the majority of the atoms is, however, determined by a potential which is rather close to a square well potential. In this case the harmonic restoring force of Eqn. (2) has to be modified. The case of a square well potential is approximated in [16] by the following expression for $\langle \Delta x^2(t) \rangle$:

$$\langle \Delta x^2(t) \rangle = \begin{cases} \langle x_a^2 \rangle t / \tau_c & \text{for } t \leq \tau_c \\ \langle x_a^2 \rangle & \text{for } t > \tau_c \end{cases} \quad (3)$$

where the correlation time τ_c equals $\langle x_a^2 \rangle \gamma / (2 k_B T)$. Here k_B is the Boltzmann constant, T is the absolute temperature and $\langle x_a^2 \rangle$ is the square of the width of the square well potential.

The elastic fraction, f_R , for Rayleigh scattering can be derived under the assumption that the total mean square displacement, $\langle x^2 \rangle$, can be separated in a vibrational part, $\langle x^2 \rangle_v$, and in a part $\langle x^2 \rangle_c$, coming from fluctuations between conformational substates. One then obtains:

$$f_R = \frac{R}{R + C} e^{-Q^2 \langle x^2 \rangle_v} \int_{-\infty}^{+\infty} g(E) dE, \quad (4)$$

where R and C are the intensities corresponding to Rayleigh and Compton process respectively. For a numerical calculation of these values compare [12]. $Q = 4\pi \sin \vartheta / \lambda$ and 2ϑ is the scattering angle. λ equals 0.86 Å. $g(E)$ is taken from [16] and corresponds to

$$g(E) = \frac{\Gamma / (2\pi)}{(E - E_a)^2 + (\Gamma/2)^2} \cdot P_0 + \frac{(\Gamma + 2a^2 \tau_c^{-1}) / (2\pi)}{(E - E_a)^2 + (\Gamma/2 + a^2 \tau_c^{-1})^2} (1 - P_1), \quad (5)$$

$$P_0 = \left[\cos \left(\frac{E}{\hbar} - \frac{E_a}{\hbar} \right) \tau_c - 2 \left(\frac{E}{\hbar} - \frac{E_a}{\hbar} \right) / \Gamma \cdot \sin \left(\frac{E}{\hbar} - \frac{E_a}{\hbar} \right) \tau_c \right] \exp(-a^2 - \Gamma \tau_c / 2) \quad (6)$$

$$P_1 = \left[\cos \left(\frac{E}{\hbar} - \frac{E_a}{\hbar} \right) \tau_c - 2 \left(\frac{E}{\hbar} - \frac{E_a}{\hbar} \right) / (\Gamma + 2a^2 \tau_c^{-1}) \sin \left(\frac{E}{\hbar} - \frac{E_a}{\hbar} \right) \tau_c \right] \cdot \exp(-a^2 - \Gamma \tau_c / 2). \quad (7)$$

Here a^2 equals $\frac{1}{2} Q^2 \langle x^2 \rangle$ because in contrast to gamma resonance absorption [16] in Rayleigh scattering the scattering angle 2ϑ has to be taken into account. E_a and Γ are the center of the resonance energy and the line width of the Mössbauer absorber, respectively. The damping constant γ of Eqn. (3) is assumed to be proportional to the microviscosity, $\eta(T)$, of the part of the molecule surrounding the fragment under consideration.

$$\gamma = \gamma_0 \exp[\varepsilon / (k_B T)] = 6\pi b \eta(T). \quad (8)$$

ε is an activation energy for the fluctuation process within the molecule. γ_0 is a temperature independent parameter which has to be fitted to the experiments and b is a linear size of a moving fragment.

The results of a fitting of Eqn. (4) to the experimental results are shown in Fig. 1 as solid lines. The integration of Eqn. (4) was performed numerically.

Unfortunately the experimental accuracy of the present measurements was not sufficient to allow to get a definit conclusion on the linewidth. Within our experimental error no line broadening was observed in the measured temperature range. However, this can come from the fact that the $K_4\text{Fe}(\text{CN})_6$ absorber used in the line width determination was rather thick and gave together with the source an experimental line width of 0.8 mm/s in a conventional Mössbauer experiment.

Good agreement between theory and experiments is obtained for the lyophilized, the crystalline and the myoglobin prepared at $p/p_s = 0.96$. The agreement is not so good in the case of Mb solution, especially at temperatures above 260 K. This is probably due to the contribution of diffusional motions. The results of our fits are summarized in Table I.

Table I. Activation energy and width of the square potential well for different Mb samples. γ_0 was taken in all fits equal to 1.66×10^{-9} [g/s].

Sample	ε [kcal/mol]	$\sqrt{a^2}$ [Å]
a) Mb at $p/p_s = 0.37$	—	0.32
b) Mb at $p/p_s = 0.94$	5.5	1.41
c) Mb solution	5.5	2.24
d) Mb crystals	6.5	0.89

Dry myoglobin ($p/p_s = 0.37$) shows practically no fluctuations. Therefore no definite value of activation energy ε could be determined. In the case of watercovered myoglobin the activation energy for the fluctuation between conformational substates is rather independent of the amount of water surrounding the molecule. This result has to be compared with recent investigations of the mobility of water by measurements of the dielectric constant [18]. It was found that the temperature dependence of the mobility of the water at the surface of a protein correlates with the mobility of iron inside the protein as determined by Mössbauer spectroscopy. The mobility inside the protein seems to be controlled by the water molecules by mechanical or electrical interactions. These forces are independent of the total amount of water surrounding the protein, as long as the water layer is not too thin. In this picture the activation energies of all water covered proteins with a polar surface should be rather similar.

In contrast to that, the average volume in which Brownian motions take place, characterised by the

linear parameter $\sqrt{a^2}$, varies drastically from sample to sample. The lowest value is obtained in the case of crystalline material. Although the water content of the sample at $p/p_s = 0.94$ is lower as in the case of the crystalline sample, $\sqrt{a^2}$ is higher. This indicates that within a crystal the dynamics of the protein are hindered by the close packing of the molecule. Protein dynamic investigations performed in crystals have therefore to be interpreted with care.

It is interesting to compare our RSMR data with the kinetic data on ligand binding to the active center of myoglobin [19]. Apparently at temperatures above 230 K the kinetic of the binding of CO or O₂ changes. While at lower temperatures the behaviour is nonexponential it becomes exponential above 230 K. In [19] this is explained by the possibility of transitions between conformational substates at higher temperatures. From Fig. 1 it is seen that above 230 K in all samples of myoglobin which are covered by water, fluctuations between conformational substates begin to play an important role.

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